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EFFECTS OF MICROWAVE RADIATION ON CELLS IN TISSUE CULTURE.(U)
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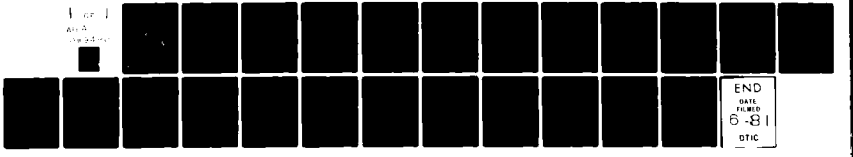

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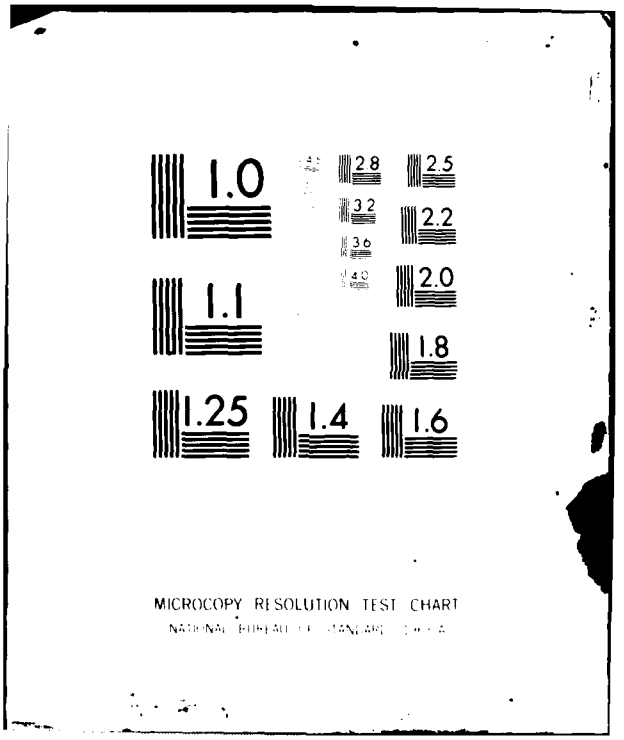
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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

The fluid-filled waveguide exposure chamber with a micropipette sample holder permits the examination of biological effects of microwave radiation at the molecular and cellular levels. As experience improves the apparatus it will become an ever more powerful tool for the investigation of the "nonthermal" and thermal interaction mechanisms of microwave-induced biologic changes in living organisms.

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FINAL REPORT

Contract No. N00014-77-C-0433

Project Title: "Effects of Microwave Radiation on Cells in Tissue Culture"

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This study has examined the capability of a fluid-filled waveguide system for investigating the effects of microwave radiation on cultured cells with particular emphasis on the cellular and molecular consequences resulting from the exposure of cells or isolated deoxyribonucleate to microwaves and the possible long-term genetic implications.

SUMMARY

A fluid-filled waveguide-exposure chamber is developed for studying effects of microwave radiation on cells *in vitro*. The system with a micropipette sample hold may be used as a prototype to isolate the apparent nonthermal factor of microwaves on cells in culture from those effects resulting from cell temperature rise. This system also allows more precise calibration of incident and absorbed microwave energies.

Compared with control, somatic cells of the Chinese hamster exhibited a lower rate of growth and difference in morphology after 2450 MHz microwave radiation for 20 minutes at a power density of 500 mW/cm^2. With the same microwave treatments, the cultured lymphocyte cultures of human did not show a significantly increased sister chromatid exchange frequency. As shown by the transformation experiments, however, there was a 2.59-8% loss in the biological activity of isolated DNA from *Bacillus subtilis* noted following microwave exposure. This observation provides an evidence for a possible genetic effect of microwave radiation at the molecular level.

I. INTRODUCTION AND RATIONALE

While there is a rich body of literature pertaining to electromagnetic effects on various tissues and organs of the body, relatively limited studies have been directed toward the effect of microwave radiation on living cells. Experiments with chick embryos using different wave frequencies to produce abnormalities were first reported by Osborne (1958) and later by Van Ummerson (1961); these studies impinge, in a general way, on living cell research. Comparable developmental abnormalities ascribable to microwave radiation were also reported by Carpenter and Livstone (1971) in early pupae of the mealworm beetle exposed to 10 GHz CW radiation in a waveguide. In their experiments, over 50% of the pupae in the irradiated population developed abnormally and in half of the abnormal animals, the front half had undergone metamorphosis to form a normal beetle head and thorax but the hind part remained in the pupal state. This demonstrated the possibility of selective absorption of the radiation flux by different parts of the pupae, or a specific teratogenic effect. On the other hand, McRee et al. (1975) exposed quail eggs four hours per day during the first five days of incubation at 2450 MHz and 30 mW/cm^2 with an absorbed energy of 14 mW/gm, but did not observe any influence on the cellular differentiation processes that occur during embryonic development. As these experiments were performed under different conditions and with different material than those used by Carpenter and Livstone, direct comparison of the results are not possible. In both cases, further investigation is warranted in order to verify validity of the observations.

A few studies on microwave-induced cytogenetical changes in animal and human cells have been reported. Janes et al. (1969) observed an increase in frequency of chromosome stickiness in cells from the bone marrow of Chinese hamster 3-5 hours following 2450 MHz irradiation. In their experiments the animals were indiscriminately subjected to field of unknown intesntiy and, therefore, a meaningful interpretation of the results cannot be made. The presence of chromosome aberrations in human cell cultures at extremely low-field levels was also reported by Guru et al. (1975). Again, these experiments lack the quantitative rigor necessary to interpret any genetic effects.

Other aspects of microwave effect on cell suspension in vitro have been studied at different levels. The observed cellular changes occurring at low incident power conditions have involved the formation of atypical, malformed cells (Valtonen, 1966), lymphoblastoid transformation (Stodolink-Baranska, 1967), inhibition of cell growth (Heller, 1970) and increased membrane permeability (Szmigielski et al., 1975). Electromagnetic radiation has also been reported to either increase, decrease or inhibit the synthesis of both DNA and protein in proliferating cells (Webb, 1975). However, to date, most experiments involving cell cultures have utilized standard, off-the-shelf containers (such as the petri dish, test tube, vial or flask) and conventional exposure techniques (such as irradiation in air-filled waveguides and open space conditions). These attempts possessed inherent dosimetry difficulties. In the majority of cases the amount of absorbed energy could not be determined. Consequently, the quantity of energy converted to cell temperature elevation could not be eliminated and, thus, the biologic effects due to any direct nonthermal interaction could not be meaningfully studied.

With the above considerations, during the past three years we have used a bioengineering approach to develop a technique for investigating the effects of microwave radiation on the in vitro cell system by using a waveguide chamber in conjunction with a micropipette sample holder. The major facets of our investigations are stated in this report.

II. THE WAVEGUIDE EXPOSURE SYSTEM

The waveguide exposure chamber used throughout this study is illustrated in Figure 1. The chamber is filled with a phosphate buffered saline (PBS) solution that is matched to air using a quarter-wavelength matching layer. The complex permittivity $\varepsilon = \varepsilon_o (\varepsilon' - j\varepsilon'')$, loss tan $\delta = \varepsilon''/\varepsilon'$ and attenuation constant of PBS (temperature = 37°C, frequency = 2450 MHz) are determined by using the perturbation technique (Harrington, 1961) as follows:

$$\varepsilon' = 61.45,$$

$$\varepsilon'' = 16.47,$$

$$\tan\delta = 0.268,$$

$$\gamma = \omega\varepsilon_o\varepsilon'' = 2.209\zeta$$

$$\alpha = 53.43 \text{ Np/m} = 0.5343 \text{ Np/cm},$$

where $\omega = 2\pi f$ is the angular frequency and,

$\varepsilon_o = 8.854 \times 10^{-12}$ F/m is the permittivity of free space.

The buffer solution is passed through a constant-temperature circulator, which maintains the culture at a desired temperature of 37°C. The unique feature of this system is that instead of a petri dish or flask, a 100 µl micropipette is utilized to hold the suspension of cells. The micropipette has an outer diameter of 1.75 mm and an inner diameter of 1.48 mm. Therefore, the wall thickness of the pipette is only 0.27 mm. Since the micropipette is a smooth cylinder whose diameter is small compared to wavelength and since the PBS (density ρ = 1.0095) that is used as the cell-suspension medium is homogeneous with that in the chamber, the absorbed energy inside the pipette is also the same as that in the buffer solution.

Because the microwave field in a waveguide is determined, the power density (P_i in mW/cm^2) of incident radiation and the rate of energy absorption (Pa in mW/g) can be calculated exactly from the following equations (Courtney et al., 1975):

$$P_i = (W_i - W_r) \exp(-2\alpha z)/A \text{ and } P_a = 4\alpha P_i/\rho$$

where W_i and W_r are incident and reflected power, respectively; α is the attenuation coefficient; z is the distance from the interface of matching layer and PBS to the position of the sample holder; and A is the cross-sectional area of the waveguide.

A block diagram of the experimental setup is shown in Figure 2. Microwave energy is derived from a MW/225 diathermy unit and is coupled to waveguides by coaxial-waveguide adaptors. A directional coupler and a Hewlett-Packard 432-A power meter with a thermistor mount (HP478-A) are used to measure forward and reflected powers. By using the described system the reflection coefficient at different power densities were determined under the air-filled and fluid-filled environments, respectively. The reflected power was found to be less than 1.5% of the forward power at all times when the waveguide exposure chamber was filled with buffer solution, as is shown in Figure 3. The temperature of the solution was closely controlled by using a constant temperature circulator, which is composed of an automatically controlled heater/compressor. Cooling capacity of the unit is 1450 BTU per -10°C. The circulator is also equipped with duplex pumps that allow PBS circulation through the microwave exposure chamber by positive and negative pressure. The rate at which the buffer solution was circulated was continuously regulated by a float-operated valve, that increased or decreased flow of PBS in order to maintain a constant level of fluid within the chamber. Both the outlet and inlet temperatures were monitored by a thermocouple-fed, digital thermometer with an accuracy of ±0.01% of the reading. The fluid temperature in the chamber just before and immediately after irradiation could also be measured directly.

Assessment of the extent of temperature fluctuation both inside and outside the micropipette sample holder in the exposure chamber was undertaken with irradiation at different power densities. The determination was done by a PBS blank experiment in which the temperature of the PBS solution within the micropipette was closely monitored by connection of a thermocouple through one end of the pipette to a digital thermometer, which prevented direct exposure of the thermocouple to radiation. The temperature of the same buffer solution in the exposure chamber was controlled by a circulator with an automatic refrigeration unit as described above and was similarly registered on the thermometer. Under these stringent experimental confines, it was found that a temperature of 37°C could be closely maintained (within ±0.1°C) during a 20-minute exposure to 2450 MHz microwave radiation at power densitites to 500 mW/cm^2. Since we have repeatedly failed to detect any temperature fluctuation inside the micropipette exceeding 0.1°C and this residual temperature difference may be expected to reduce consistently as the diameter of the sample holder is decreased, the designed exposure chamber offers an effective approach and valuable tool for studying the effects of microwaves on cell systems. (Chen and Lin, 1976 and 1978.)

III. MATERIAL AND METHODS

Employing the above described waveguide exposure system, the molecular and cellular effects of 2450 MHz microwave radiation were examined. Biological materials used were the established somatic cell line of a Chinese hamster (V 79), primary lymphocyte cultures of human, and a bacterial strain of Bacillus subtilis (trp C2).

Transforming DNA preparations were isolated from a prototrophic wild-type strain (SB 19) of B. subtilis. The method of Marmur (1961) was adopted in the extraction of cellular DNA. It consisted of the following steps: lysis of cells with lysozyme and dupanol, deproteinization of cell lysate with a 4:1

chloroform/N-butanol mixture, precipitation of DNA with absolute ethanol, and removal of the contaminated RNA with RNase treatments.

The media for growing culture and transformation tests are as described in a previous publication (Chu and Iyer, 1973). Spizizen's minimal medium supplemented with 50 µg/ml of nine stimulatory amino acids was used for growing a culture rendered to competence for transformation. The make-up of transformation medium was essentially the same as that of the minimal medium, except for the addition of only 5 µg/ml of the nine amino acids plus L-tryptophan.

The regimen of irradiation and experimental procedures will be further detailed whenever appropriated.

IV. RESULTS AND DISCUSSION

I. Cellular Effects of Microwave Radiation

An investigation of effects of 2450 MHz microwave radiation was conducted on Chinese hamster somatic cells (V 79) by employing the designed waveguide system. The cells were routinely grown in Eagle's minimum essential medium (EME) as supplemented with 10% fetal calf serum under a humidified atmosphere of 5% CO_2. The incubation temperature was maintained at 37°C throughout. For experiments, exponentially growing cells were removed from plastic tissue culture bottles by means of trypsinization. After repeated washings with phosphate buffered saline, cells were collected by centrifugation and resuspended with PBS in a sterile vial. Aliquots of cell suspension were immediately transferred to a micropipette sample holder (1.75 mm in diameter) and were then subjected to a 1059 mW/g dose of microwave irradiation (50mW/cm^2 power density). The exposure duration was 20 minutes. The regimen and conditions for sham-exposing control cells were exactly the same as those of irradiated cells, except that microwave radiation was not applied. After treatment, both the irradiated and control cells were plated onto petri dishes and allowed to develop into colonies. The cytotoxic effects of radiation were assayed over a 12-day incubation period in terms of cellular growth rate, morphologic change, and clone-forming ability (Chen, 1978).

Under these experimental confinements, the growth rate of irradiated cells decreased by nearly 30%. Further, about 10% of the irradiated cells underwent a distinct morphological transformation within 48 hours of incubation after irradiation. It can be seen in Figure 4a and b that control cells mitosed characteristically with an average generation time of about 12 hours, while the irradiated cells divided at a much slower rate. At this stage, cell vacuoles were first observed in the cytoplasmic compartments, which gradually increased in size. The irradiated cells then showed enlarged volume and irregularity during continued growth. Subsequently, star-shaped, giant-ruffle cells appeared. With continuing incubation of the cells up to 12 days at 37°C, the microwave-induced transformants finally exhibited a fibroblast type of growth. The cells were elongate and proliferated in matched parallel position to form small, irregular single-layered masses of cells over the surface of a plate (Figure 5a). Such observed cellular abnormality appeared to be irreversible. This is at variance with the growth pattern revealed in the unirradiated cell culture in the control. Normal

cells not only divided at a significantly faster rate but they grew in relatively compact fashion with no particular orientation, forming multi-layered colonies in petri dishes (Figure 5b).

The intrinsic nature of morphological changes induced in the Chinese hamster somatic cells by microwave irradiation was then investigated. In view of the reports by others that cells transformed by carcinogens, viruses and ionizing radiations usually resulted in alteration of cellular biochemistry and the eventual surface properties of a cell, our attempts were diligently made to examine the possible involvement of cellular nucleotide metabolism in the onset of morphological transformation. To achieve this research goal, we have introduced the use of a potent chemical inhibitor involved in the nucleotide pathway into the experiments. It is known that theophylline is effective in inhibiting endogenous phosphodiesterase activity and generally causes a significant rise in the level of cAMP in cells. Hence, the presence of a critical quantity of exogenous theophylline in a cell at the time of microwave irradiation should thus provide a sensitive approach for detection of the underlying microwave effect. Using a wide range of theopnylline concentrations (0.0001 M to 0.1 M), we found that with the addition of a dose of 0.001 M theophylline to the growth medium, it produced only a slight influence in the growth patterns of cells in vitro; but, most cell colonies after a 3-day incubation period exhibited a state of normal division cycle indicating an attainment of the threshold concentration of theophylline (Table 1). By administering this theophylline concentration, we, however, repeatedly failed to detect any enhancement of cytotoxic action following irradiation of the cells to different power intensities of microwaves. These findings could be taken to suggest that the cellular cAMP level is probably not directly responsible.

Although mechanisms of the observed morphologic difference between control and irradiated cultures under our controlled temperature condition is not known, the possible factors (not necessarily exclusive in operation) should be considered. As 37°C rigidly maintained throughout the experiment (within ±0.1°C) is the optimal temperature for growth of the mammalian cell line under in vitro conditions and as it was observed that experimental temperature variance of 1°C above or below optimal did not alter the amount of morphologic difference, it is quite conceivable that a truly athermal influence, akin perhaps to the field-induced pearl-chain formation at a dose rate of 1,000 mW/g (Schwan and Sher, 1969) and the frequency dependence of microwave effects on Escherichia coli bacteria (Webb and Booth, 1969) is a contributing factor. Our experimental conditions, on the other hand, in maintaining culture temperature at 37°C, require that the high rate of heating be offset by an equivalent high rate of cooling; thereby, intense thermal fluxes could occur. One may also speculate that thermal microgradients may develop at any time during the course of microwave exposure and that these could be responsible for the observed cytotoxic effects. Further studies are needed to clarify these points.

2. Frequency of SCE Following Microwave Exposure

Extensive mutagenic assays by previous investigators of both known and suspected mutagens in cultured mammalian cells have shown that the effects of physical and chemical mutagens on eukaryotic chromosomes can be readily detected by sister chromatid exchanges (SCEs). Studies with known mutagens

have revealed that significant SCEs are observed by treatements at a very low dose level to which ordinary chromosome aberrations fail to respond. The high sensitivity of SCE thus recommend itself as a valuable detection system for the evaluation of any potential effects on chromosomes due to microwave radiation.

In this research, experiments were conducted to determine the effects of 2450 MHz microwave radiation on SCE frequency in cultured human lymphocytes. Peripheral blood sample was obtained in heparinized syringes, and after gravity sedimentation, 0.7 ml of lymphocyte-rich serum was added to McCoy's 5A chromsome medium containing 20% fetal bovine serum, phytohemaglutinin, and 5-bromodeoxyuridine (*BrdU*, 37 μg/ml). The lymphocytes were harvested by standardized procedures after 66 hours of incubation at 37°C. At 17 hours prior to culture harvest, the lymphocytes were treated with microwave radiation (at a range of power densities up to 500 mW/cm^2) for 15, 30, or 60 minutes. A zero minute and a 60 minute sham-treatment served as controls. Cell preparations were stained to visualized sister chromatid exchanges by a modification of the method of Goto et al. (1975). Metaphase spreads were treated with 0.5% 33258 Hoechst for 10 minutes, rinsed and mounted in pH 6.8 phosphate buffer solution, exposed to sunlight for one hour, and stained with Gurr R66 Giemsa. The experiment was duplicated on separate occasions using peripheral blood from different volunteer donors. Sister chromatid exchanges were scored in up to 40 metaphase cells per treatment. Exchanges at the centromere were also counted as SCEs. Statistical analysis of the data was carried out according to the Model I analysis of variance and are presented in Table 2. Results show that the controls in experiment 1 had from 15.1 to 15.8 SCE/cell and the microwave-irradiated cells had from 15.0 to 17.0 SCE/cell. In experiment 2, the controls had from 20.2 to 22.7 SCE/cell and the microwave-exposed cells had from 21.5 to 22.9 SCE/cell. Thus, the cultured lymphocyte cells exposed to microwave radiation for a period up to 60 minutes did not exhibit a significantly increased SCE frequency.

It should be noted that the control and treatement SCE count in experiment 1 was uniformly lower than in experiment 2. Since exposure to visible light influences SCE frequency (Wolff and Perry, 1974), the cultures were wrapped in aluminum foil for the duration of culture except during the experimental exposures and during preparation for the irradiation when the concentrated cell suspension was prepared for the micropipette. Room lights were turned out but the windows could not be shaded, so a difference in sunlight intensity could account for part of the difference in SCE frequency between the experiments. Other possible factors that might have influenced the SCE frequency in experiments 1 and 2 include a difference in baseline SCE frequency between two volunteer donors, or an inadvertent difference in the BrdU concentration in the two sets of tissue culture media.

3. Molecular Effects of Microwave Radiation

The molecular probe for ascertaining microwave effects by means of DNA-induced bacterial transformation systems has been conducted by studying the action of microwave radiation at isolated deoxyribonucleate from Bacillus subtilis and the kinetics of inactivation involved in the transforming activity of the molecules. With conventional subcritical-heat treatments, our results clearly showed that denaturation of transforming DNA was inevitably accompanied by a loss in its transforming capability (Figure 6).

In the experiments, an auxotrophic mutant of B. subtilis strain 168 (trp C2) characterized by its inability to synthesize tryptophan was used as recipient and the prototrophic wild-type strain (SB 19) was employed for the extraction of transforming DNA. Samples of DNA were treated with various doses of microwave radiation before use.

Innoculum of the recipient bacteria originally maintaied in nutrient agar was transferred to 20 ml Penassay broth in a flask and allowed to grow for 15 hours at 37°C with vigorous shaking. Cells were collected by centrifugation and resuspended in growing medium of the same volume. The growing cells were allowed to divide for a period of 4 to 5 hours at 37°C and then transferred to transforming medium for development of competence. The prepared competent culture was immediately used in transformation experiments.

For transformation tests, competent culture of B. subtilis 168 try C2 was exposed to 1.4 µg/ml of SB 19 DNA for 20 minutes at 37°C with vigorous aeration. The cells/DNA reaction was terminated by adding 10 g/ml of Mg^{++}-activated DNase into the reaction mixture and the reaction products were allowed to incubate for 10 minutes. The transformation reaction mixture was then suitably diluted and plated onto minimal and nutrient agar plates for determination of the number of Trp^{+} transformants and the total viable counts.

Results summarized in Table 3 reveal that irradiation of a non-saturating concentration of DNA (up to 1 ug/ml)with 2.45MHz microwave at 750 mW/cm^2 for 30 minutes is found to cuase a loss in the transforming capability of the donor DNA to induce genetic transformation of the recipient cells. The extent of this reduction in transformation frequency following DNA exposure to microwaves at this dose was found to be in the range of 2.59-8%. When a comparison between the irradiated DNA and a control DNA sample was made, there was found no detectable difference in the DNA's physio-chemical properties as measured by optical densities at 260 nm (Table 4). Nor was there any molecular lession discernable by means of electron microscope examinations (Figures 7 and 8).

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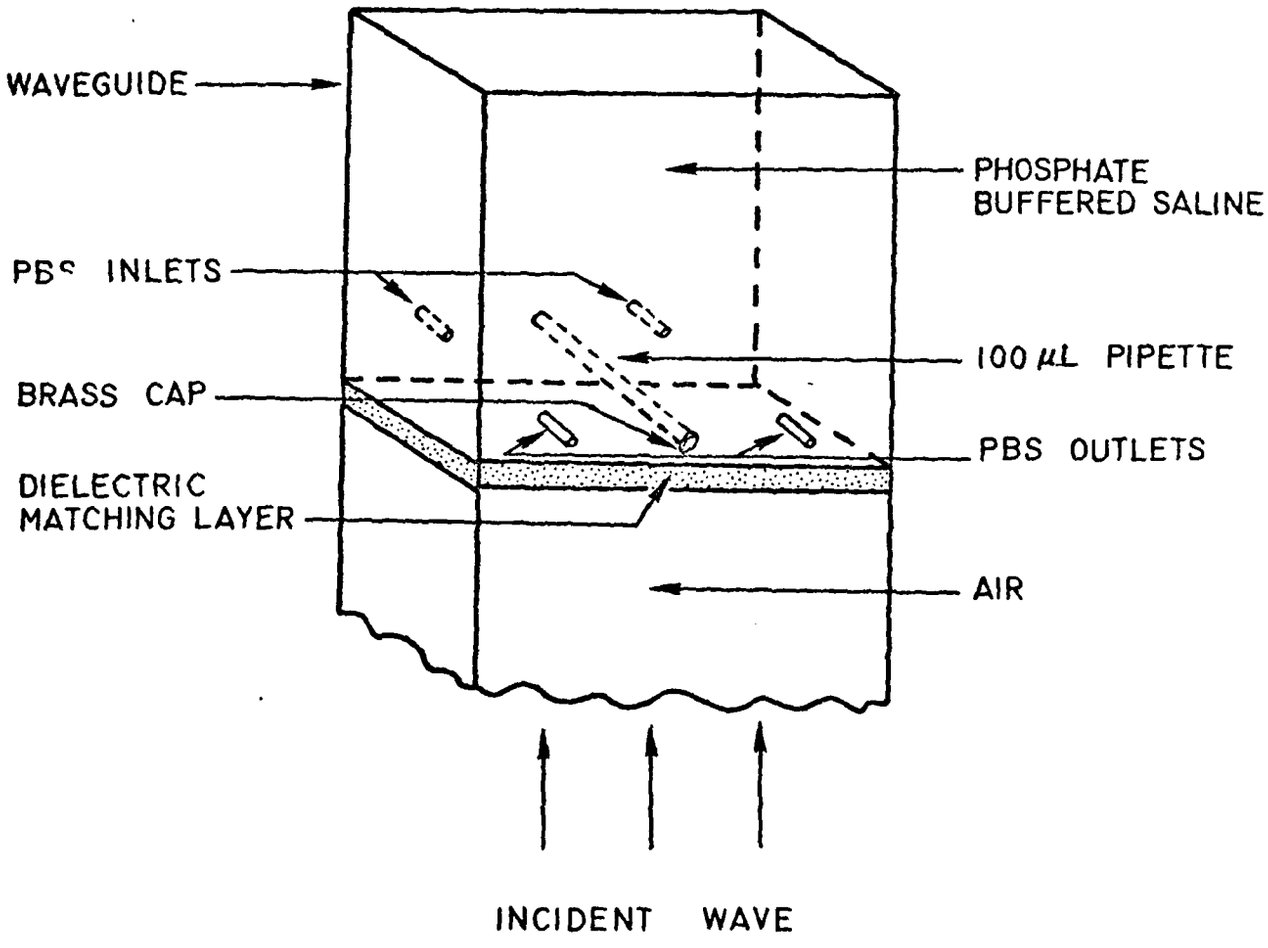


Figure 1. Structure of Waveguide Exposure System

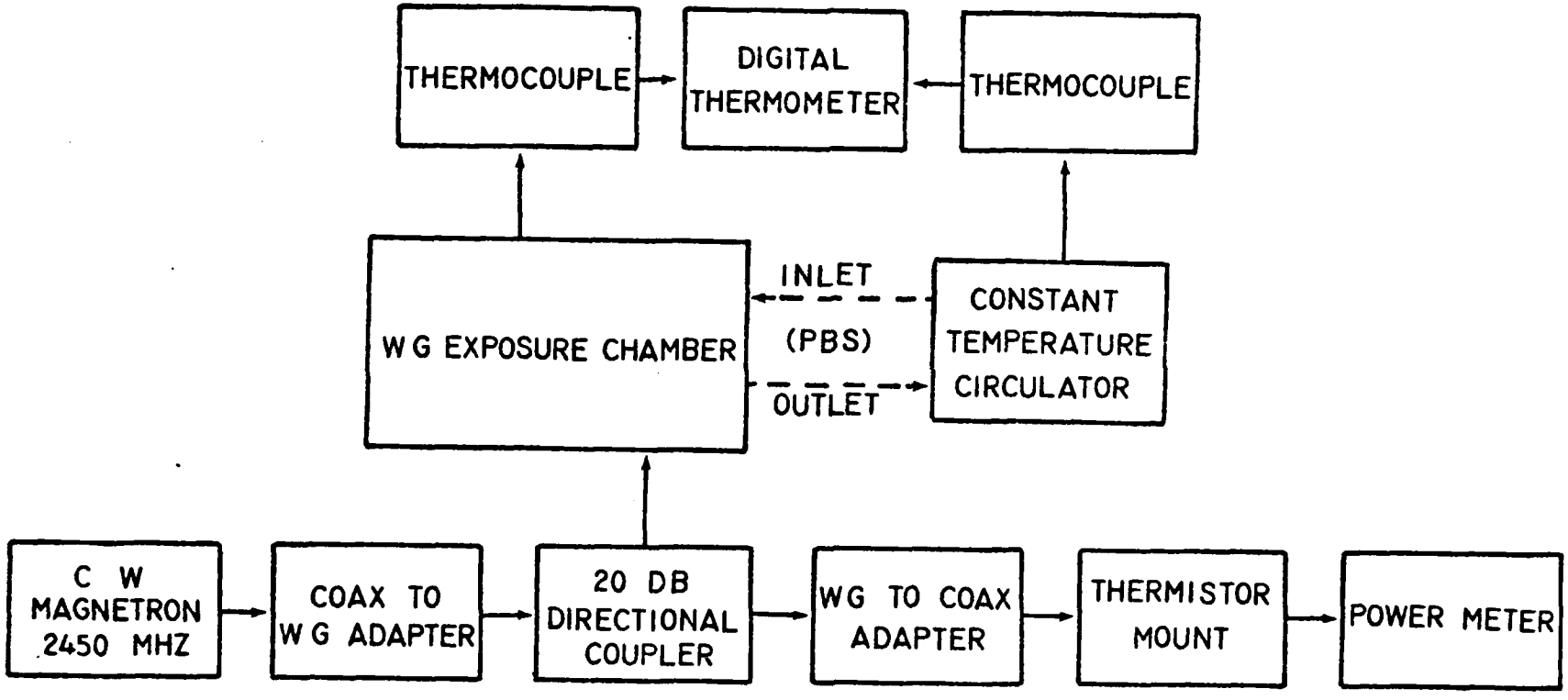


Figure 2. Block Diagram of Experimental Setup

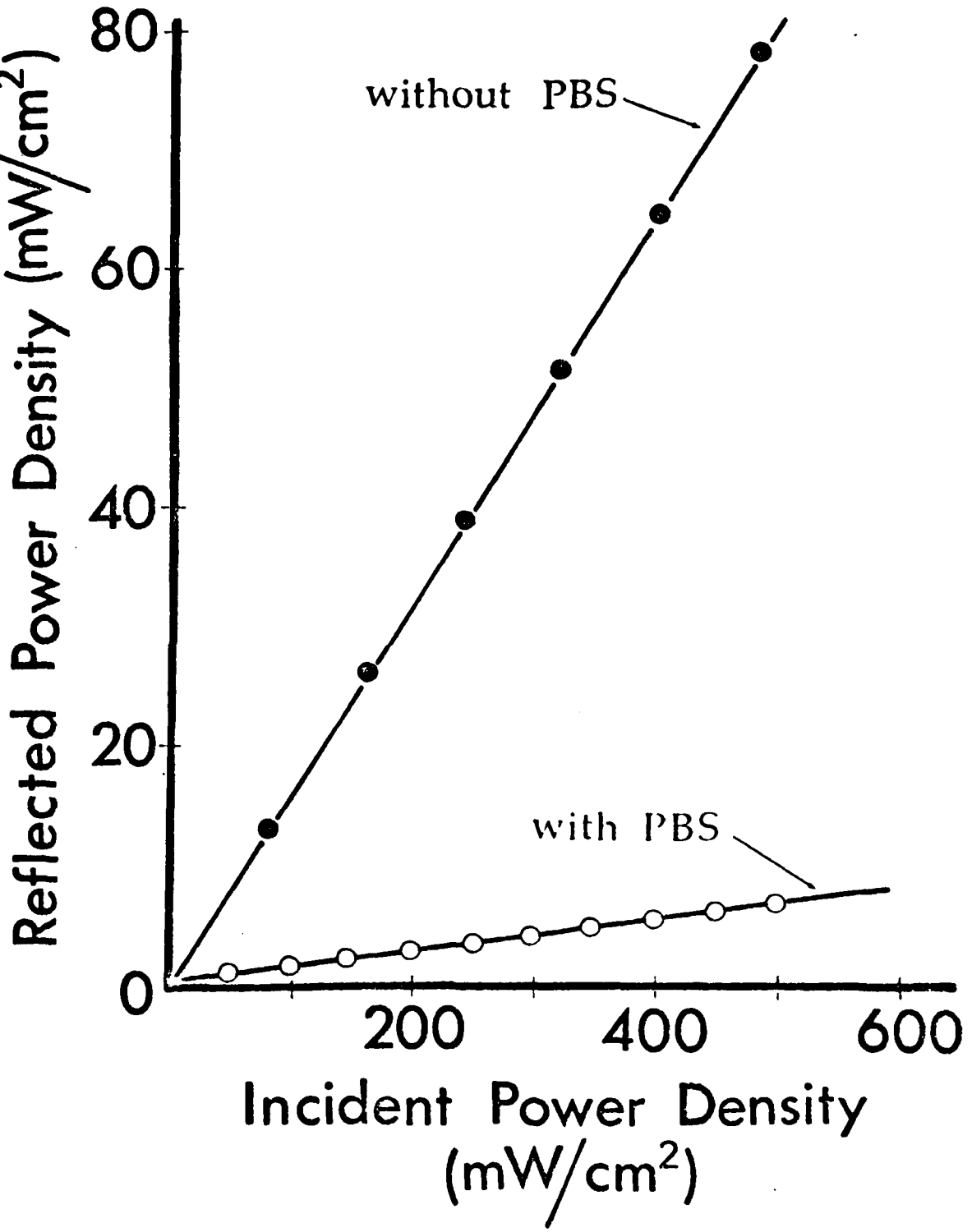


Figure 3. Reflection Coefficient of Power Density Measured under Different Exposure Environments.

Figure 4. Photomicrographs of Chinese Hamster Somatic Cells after Three Days of Incubation at 37°C. (a) Control cells are shown in log phase of growth with an average generation time of 12 hours, (b) the irradiated cells divide at a much slower rate, and the appearance of giant-ruffle cells is typical. (Both magnification X 1,600).

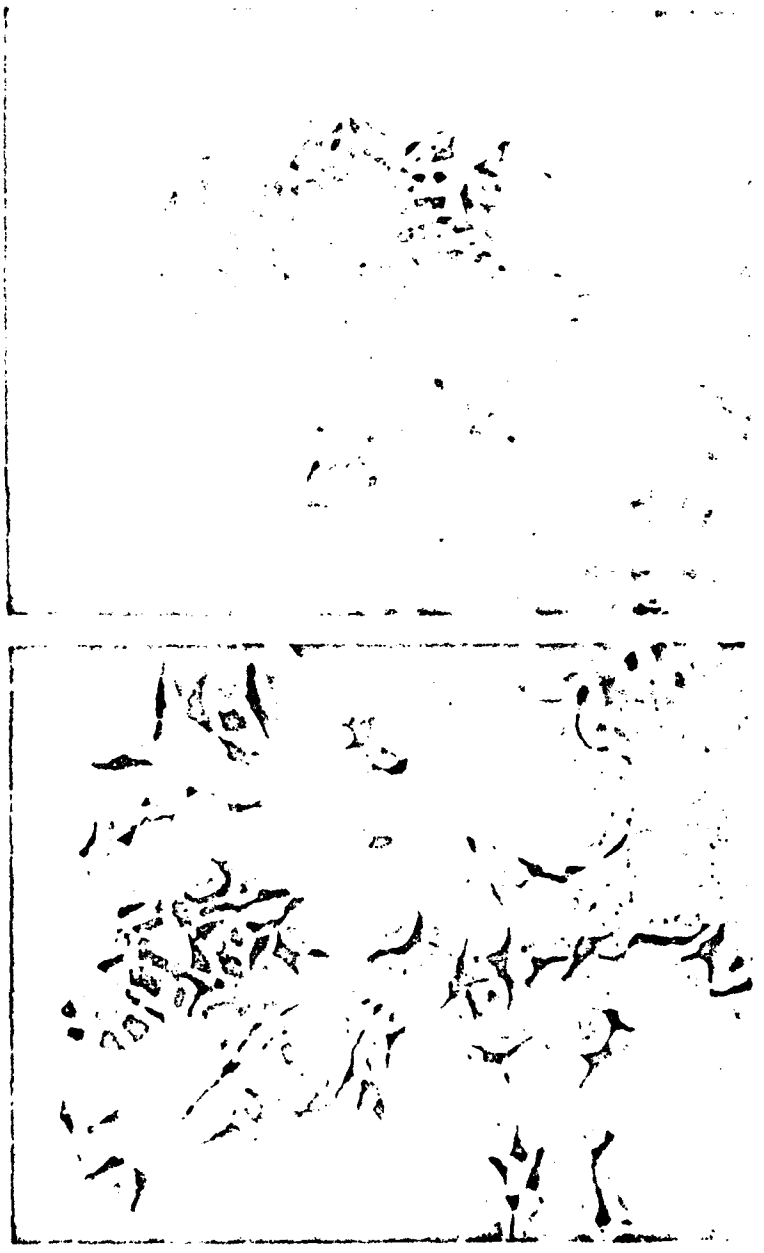


Figure 5. The Growth Patterns and Clone Morphology of Chinese Hamster Cells In Vitro. (a) Normal cells grow in a relatively compact fashion with no particular orientation, forming a multi-layered colony. (b) Microwave induced transformed cells exhibit a fibroblast type of growth and proliferate in matched parallel position to form irregular, single-layered masses of cells over the surface of a plate. (Both magnification X 300).

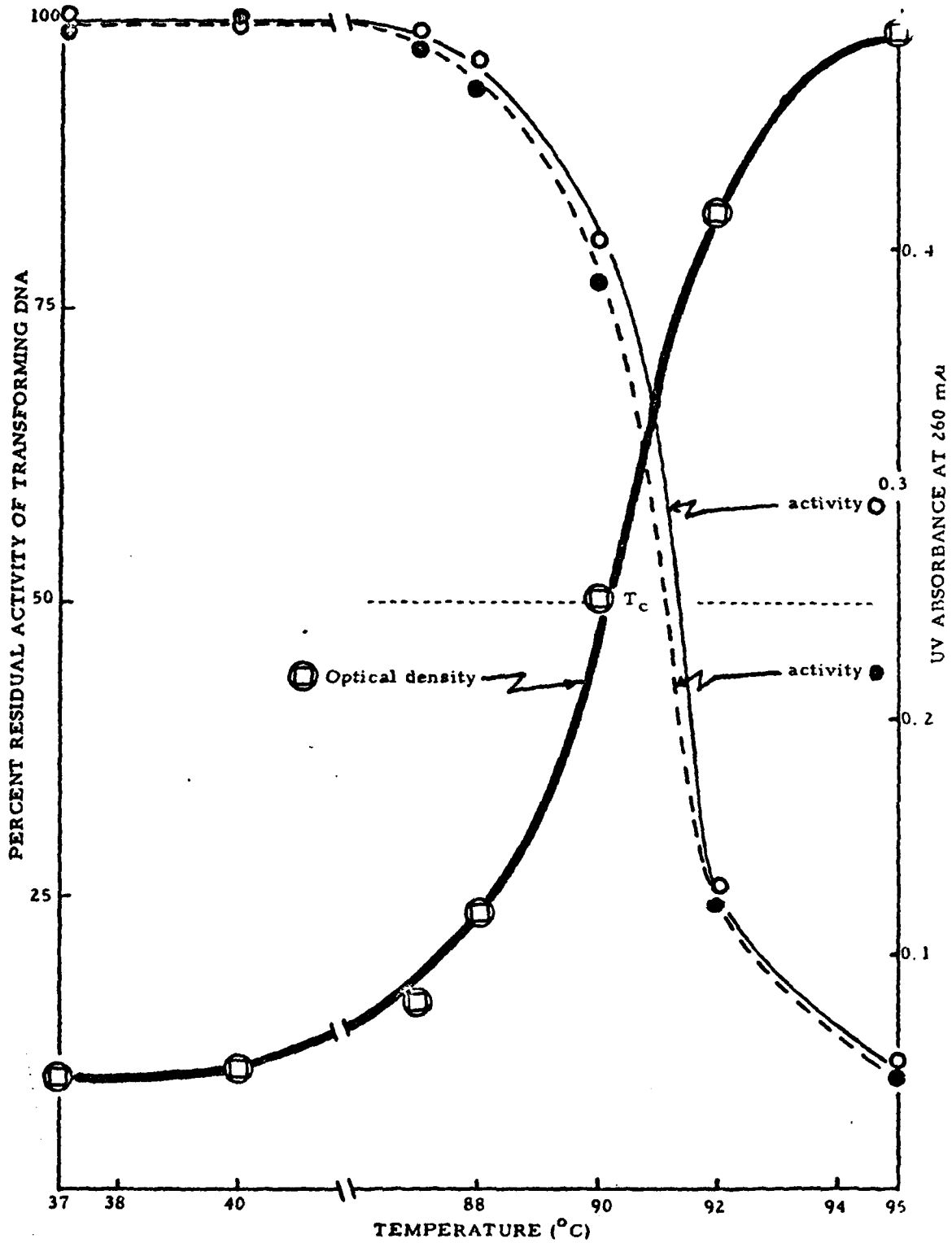


Figure 6. Residual Transforming Activity of B. subtilis DNA following Subcritical-heat Treatments.

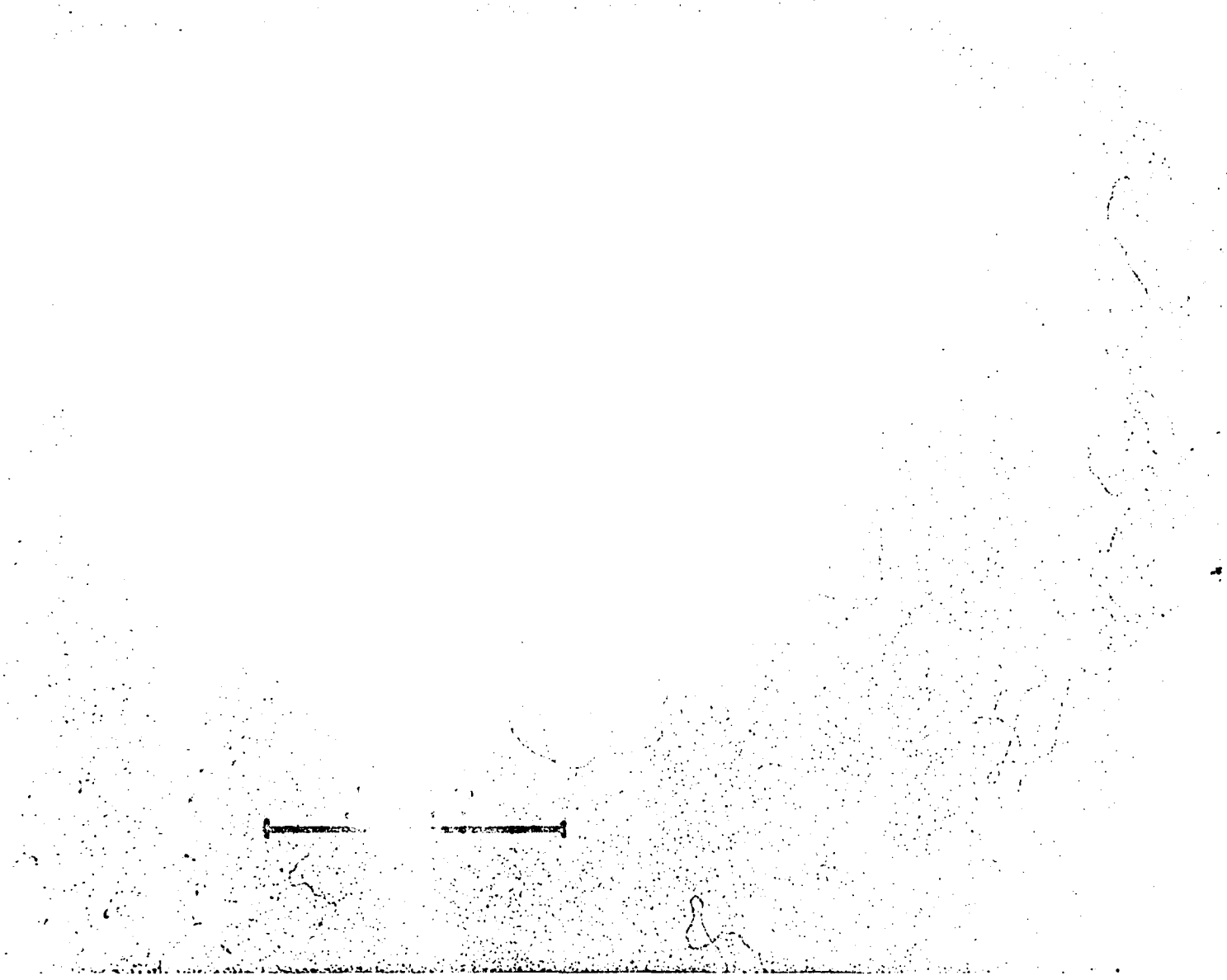


Figure 7. An Electron Micrograph of Microwave Irradiated DNA, Showing A Normal Molecular Characteristic as Compared with the Unirradiated DNA Samples.

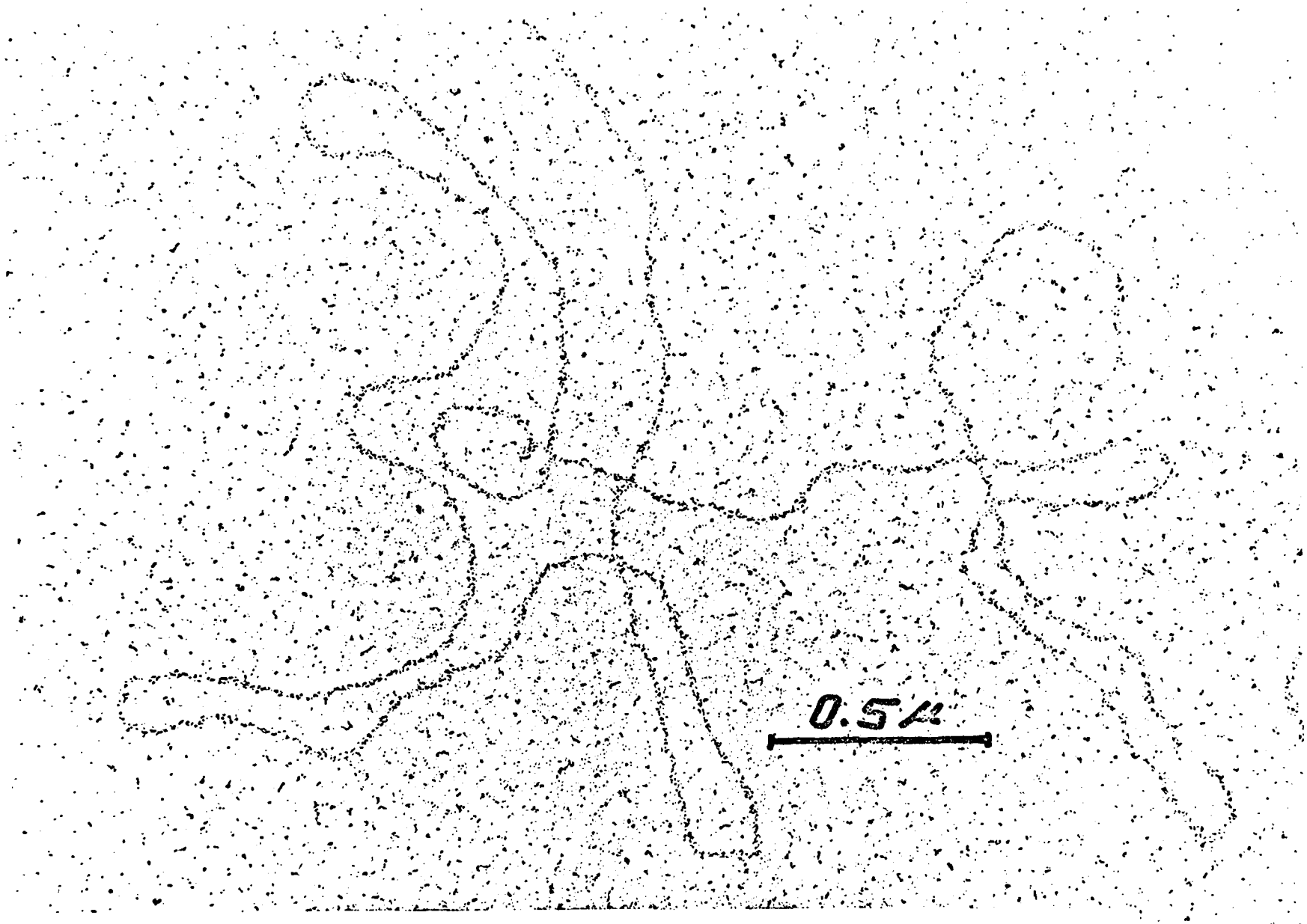


Figure 8. An Electronmicrograph of the Microwave Irradiated DNA at A Higher Magnification.

TABLE 1

Effects of Theophylline on the Growth of Hamster Cells in Vitro

Concentration of Theophylline	Growth Charateristics
0*	Normal multi-layer-colonies formed without particular orientation
0.1 M	No growth or only moribund cells formed
0.01 M	Fibroblast type of growth showing profound effects
0.001 M	shows moderate effect; but most cells are in a state of more or less normal type of division, indicating a threshold concentration of theophylline
0.0001 M	shows + or - effect

* PBS was used

TABLE 2

Sister Chromatid Exchanges of Cultured Human Lymphocytes in the Presence and Absence of Microwave Irradiation

Treatment (min.)	No. SCE/No. Cells	SCE per Chromosome	SCE/Cell Mean ± S. D.*		P
Experiment 1					<0.25
0' Sham-exposure	600/38	0.34	15.8	3.9	
60' Sham-exposure	497/33	0.33	15.1	3.2	
15' Microwaves	506/33	0.33	15.3	4.5	
30' Microwaves	598/40	0.33	15.0	3.3	
60' Microwaves	602/35	0.37	17.2	3.9	
Eperiment 2					<0.10
0' Sham-exposure	525/26	0.44	20.2	3.6	
60' Sham-exposure	499/22	0.49	22.7	5.9	
15' Microwaves	573/25	0.50	22.9	4.6	
30' Microwaves	464/21	0.48	22.1	5.8	
60' Microwaves	237/11	0.47	21.5	3.0	

*Standard deviation

TABLE 3. Effect of 2450 MHz Microwave Radiation on Transforming DNA of B. subtilis

DNA Concentration (μg/ml.)	Number of Trp^+ Transformants/ml.* Microwave Irradiation	Sham-exposure	Number of Colony Forming Unit x 10^5	Reduction in Transformation Frequency (%)
0.01	75	77	124	2.59
	70	-	122	-
0.1	784	818	131	4.15
	798	835	132	4.43
1.0	3420	3740	120	8.55
	3120	3420	109	8.77

as the experiment except microwave radiation was not supplied. Number of transformant represents the average value of five plating counts.

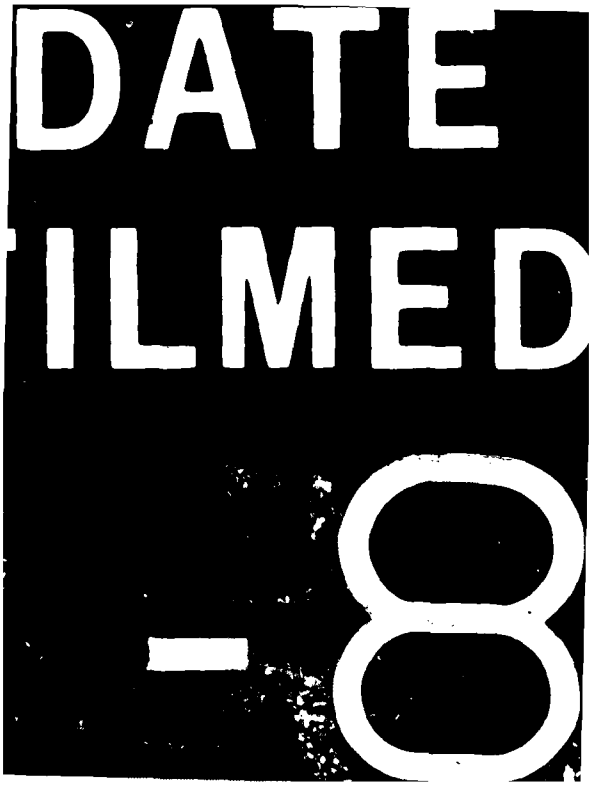
TABLE 4

Spectrometric Characterization of Transforming DNA

After 2450 MHz Microwave Irradiation*

Duration of Microwave Irradiation	Relative Absorbance at 260 nm
10	0.507
15	0.505
20	0.507
25	0.508
30	0.507

*with a power density of 500 mW/cm^2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